UNIVERSIDADE FEDERAL DE SANTA CATARINA

CURSO DE GRADUAÇÃO EM FISIOTERAPIA

CITRAL INIBE A INFLAMAÇÃO AGUDA E NOCICEPÇÃO EM CAMUNDONGOS: O PAPEL DOS RECEPTORES TLR4 E TLR2/DECTINA-1

ELAINE CRISTINA DALAZEN GONÇALVES

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Trabalho de conclusão de curso de graduação apresentado à disciplina TCC II do Curso de Fisioterapia da Universidade Federal de Santa Catarina, como requisito parcial para obtenção do título de Bacharel em Fisioterapia.

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RESUMO

Citral (3,7-dimethyl-2,6-octadienal), um monoterpeno de cadeia aberta e principal componente bioativo do capim limão, apresenta diferentes propriedades farmacológicas, tais como: *i)* inibidor da atividade oxidante de células apresentadoras de antígenos (APCs), tais como os macrófagos; *ii)* inibidor da ativação do fator de transcrição pró-inflamatório NF-κB; *iii)* inibidor da expressão da enzima ciclooxigenase do tipo 2 (COX-2); e *iv)* ativador do fator de transcrição pró-resolução receptor proliferador de peroxissomo (PPAR)-α e γ. Além disso, o citral atua como agonista inverso dos canais iônicos e receptores de potencial transitório (TRPs) expressos em neurônios sensoriais (TRPV1, TRPV3, TRPM8 e TRPA1), produzindo uma inibição de longa duração de TRPV1–3 e TRPM8, demonstrando potencial analgésico em diferentes tipos de dor. No entanto, até o presente momento, não existem relatos dos efeitos antiinflamatórios e analgésicos de citral em modelos inflamação aguda, assim como a sua interação com a produção de eicosanóides e modulação dos receptores do tipo *Toll-like* (TLR). Neste trabalho demonstramos que o tratamento oral com citral (nas doses de 50 – 300 mg/kg) significativamente inibiu o edema de pata e a alodinia térmica induzidos pela carragenina. Além disso, o pré- tratamento com o citral inibiu a resposta inflamatória induzida por LPS e zimosan, ligantes dos receptores TLR4- e TLR2/dectina-1, respectivamente. Por fim, nossos resultados demonstram os efeitos antiinflamatórios e analgésicos do citral, os quais parecem estar relacionados à modulação dos receptores da imunidade inata, tais como o TLR4 e o TLR2/dectina-1.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

PRRs Germline-encoded pattern-recognition receptors
PAMPs Pathogen-associated molecular patterns
DCs Dendritic cells
TLRs Toll-like receptors
TIR Toll/interleukin-1 receptor
IFN Interferon
NO Nitric oxide
COX Cyclooxygenase
5-HT Serotonine
BK Bradykinin
PGs Prostaglandins
TNF-α Tumor necrosis factor alpha
IL-1 Interleukin- 1
IL-6 Interleukin – 6
O_{2-} Superoxide anion
LPS Lipopolysaccharide
Myd88 Myeloid differentiation marker MyD88
TIRAP TIR domain-containing adaptor protein
TIRAP/Mal TIR domain-containing adaptor protein - MyD88 adapter-like
TRAM TLR adaptor molecule
PPAR-α Activated peroxisome proliferator-activated receptor
PPAR-ɣ Activated peroxisome proliferator-activated receptor
NF-Kb Nuclear factor kappa B
I.p Intraperitoneally
I.pl. Intraplantar
NaCl Sodium chloride
µl Microliter
µg Microgram
MD-2 Myeloid differentiation factor 2 (MD-2)
CD14 Cluster of differentiation 14
ALI Acute lung injury
iNOS Nitric oxide synthase

Citral reduces acute inflammation and nociception in mice: the role of TLR4 and TLR2/dectin-1 pathways

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Abstract

Citral (3,7-dimethyl-2,6-octadienal), a bioactive component of lemongrass, can inhibit macrophage oxidant activity, and NF-κB activation, as well as COX-2 expression and activation peroxisome proliferator-activated receptor (PPAR)-α and γ. In addition, citral activates TRP channels in sensory neurons (TRPV1 and TRPV3, TRPM8, and TRPA1), and produces long-lasting inhibition of TRPV1–3 and TRPM8, while transiently blocking TRPV4 and TRPA1, demonstrating analgesic potential for allodynia and other types of pain. However, there are no reports about the anti-inflammatory and analgesic effects of citral in induced inflammation by carrageenan- and TLR-depend pathways and algesic response. In this study, we investigated the effect of citral in experimental models of acute inflammation and nociception in mice. Oral treatment with citral (50 – 300 mg/kg) significantly inhibited carrageenan-induced paw edema and thermal allodynia. Furthermore, citral also modulated the inflammation induced by LPS and zymosan, a TLR4- and TLR2/dectin-1 ligand, respectively. Our results demonstrate that oral citral treatment displayed anti-inflammatory and analgesic effects, and these effects seem to be related to its TLR4 and TLR2/dectin-1 modulation.

Keywords: inflammation; acute pain; carrageenan; TLR; citral.

Sumário

1. Introduction

Inflammation is a physiological phenomenon that operates during severe perturbations of homeostasis, such as infection and injury, triggered by innate immune receptors that recognize pathogens and damaged cells (Noah, 2012). Recognition of microbial pathogens is an essential element for the initiation of innate immune responses such as inflammation, which is mediated by germline-encoded pattern-recognition receptors (PRRs) that recognize molecular structures that are broadly shared by pathogens, known as pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Geremia et al., 2014). Upon PAMP recognition, PRRs initiate a serious of signaling programs that execute the first line of host defensive responses necessary for killing infectious microbes. Moreover, PRR signaling simultaneously induces maturation of dendritic cells (DCs) and, hence, activate adaptive immunity (Kawai and Akira, 2011). Toll-like receptors (TLRs), the first PRRs to be identified, are type I transmembrane proteins and comprise an ectodomain - that mediate the recognition of PAMPs - a transmembrane region, and cytosolic Toll-IL-1 receptor (TIR) domains - that activate downstream signaling pathways. To date, 10 and 12 functional TLRs have been identified in human and mouse, respectively (Akira et al., 2006; Park and Lee, 2013). TLR signaling pathways are finely regulated by TIR domain-containing adaptors, such as myeloid differentiation (MyD) marker MyD88, TIRAP/Mal, TRIF and TRAM whose activation initiates downstream signaling events that directs to the secretion of inflammatory mediators, such as type I IFN, chemokines, nitric oxide (NO), cyclooxygenase (COX) and antimicrobial peptides (Kawai and Akira, 2010).

Carrageenan is a generic name for a family of gel-forming and viscosifying polysaccharides, which are obtained by extraction from edible red seaweeds – mainly Rhodophyceae – and is a widely used test to determine anti-inflammatory activity and constitutes a simple and routine animal model for evaluation of pain at the site of inflammation without any injury or damage to the inflamed tissue (Seibert et al., 1994; Portanova et al., 1996; Zacharopoulos and Phillips 1997; Morris, 2003). The injection of carrageenan causes edema, increase tissue volume, and exacerbated sensitivity to thermal and mechanical stimuli (Chan et al., 1995; Khanna et al., 1997; Riendeau et al., 1997; Zhang et al., 1997). There are several mediators involved in carrageenan-induced inflammation. Histamine, serotonine (5-HT) and bradykinin (BK) are the first detectable mediators in the early phase of carrageenan-induced inflammation; prostaglandins (PGs), produced by COX pathway, are involved in the increased vascular permeability and are detectable in the late phase of inflammation. Local and/or systemic inflammation is associated with enhanced levels of the pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 (Cuzzocrea et al., 1999; Morris, 2003). Local neutrophils infiltration and activation also contribute to this inflammatory response by producing, among other mediators, oxygen-derived free radicals such as superoxide anion (O_2^-) and hydroxyl radicals (OH^-) (Posadas et al., 2004).

Lipopolysaccharide (LPS), a bacterial endotoxin and TLR4 agonist, induces upregulation of proinflammatory cytokines by immune cells, including macrophages and DCs, thereby mimicking the innate immune response to bacterial infection (Laugerette et al., 2011; Manco et al., 2010; Rietschel et al., 1994), and can serve as a short-term model for investigating the actions of

different classes of anti-inflammatory and analgesic drugs (Kanaan et al., 1997; Mehta et al., 2010; Sun et al., 2010). Currently, TLR4 signaling has been divided into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. Based on studies using MyD88-deficient macrophages, the MyD88-dependent pathway was shown to be responsible for proinflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of Type I interferons and interferon-inducible genes (Lu et al., 2008; Park and Lee, 2013).

Zymosan, a polysaccharide from the cell wall of *Saccharomyces cerevisiae*, is composed primarily of glucan and mannan residues (Brown et al., 2002; Di Carlo, 1958; Reid et al., 2004). The recent discovery of PRRs and their role in innate immunity has led to a re-evaluation of our concepts of zymosan-induced inflammation (Gantner et al., 2003; Medzhitov, 1997). TLR2 is a receptor for zymosan, acting in collaboration with CD14 and TLR6 (Gantner et al., 2003; Ozinsky, 2000; Underhill, 1999). Thus, ligand binding to TLR2 induces the activation of transcriptional factor NF-κB with consequent production of inflammatory mediators, for instance IL-1β, IL-6 and IL-8, as well as the expression of the co-stimulatory molecule, like B7.1 (Medzhitov, 1997; Reid et al., 2004). Additionally, zymosan is able to induce maturation of DCs and to stimulate their production of IL-2, providing evidence for an essential link between the innate and the adaptive immune responses (Granucci, 2003; Roitt, 2001).

Citral (3,7-dimethyl-2,6-octadienal) (Fig. 1A), a monoterpene compound, is a mixture of tautomers geranial (trans-citral) and neral (cis-citral) found in the essential oils of numerous medicinal plants, especially, *Cymbopogon citrates* -

known as „„lemongrass"". Lemongrass is a widely used plant, particularly in Southeast Asia and Brazil, where it is used as a food flavoring, as a perfume, and for its medicinal properties. Tea or essential oil from lemongrass is used as analgesic, anti-inflammatory and diuretic (Ferreira, 1984; Nishijima et al., 2014). Several studies have reported the medicinal use of *Cymbopogon citrates* and Citral, although little is known about its effect on the immune system.

A very recent study conducted by Katsukawa and colleagues showed that Citral suppressed COX-2 expression and activated peroxisome proliferator-activated receptor (PPAR)-α and γ. In addition, PPARγ-dependent suppression of COX-2 promoter activity was observed in response to citral treatment, and in human macrophage-like U937 cells, citral suppressed both LPS-induced COX-2 mRNA and protein expression, dose-dependently (Katsukawa et al., 2010). Earlier studies have demonstrated that Citral acts as a partial agonist of transient receptor potential (TRP) channels; it is useful for allodynia and other types of pain (Stotz et al., 2008). Furthermore, Citral has been shown to inhibit oxidant activity (Cheel et al., 2005, Barroso et al., 2011) and nitric oxide (NO) production (Lee et al., 2008), macrophage activation, NF-κB activation, and cytokine production (Bachiega et al., 2011). However, to our knowledge, there are no reports on the anti-inflammatory and analgesic effects of Citral in carrageenan- and TLR-depend pathways induced inflammation and algesic response. Keeping the above data in mind, the purpose of the present study was to investigate the effect of citral in experimental models of acute inflammation and nociception in mice.

2. Methods

2.1. Experimental animals

Experiments were conducted using male Swiss mice (25 - 40 g) obtained from the Universidade Federal de Santa Catarina. The mice were kept in groups of four to six animals per cage, maintained under controlled temperature (22 ± 2°C) with a 12 h light/dark cycle (lights on at 07:00 h) and were given free access to food and water. All the procedures used in the present study were approved by the Institutional Ethics Committee of the Universidade Federal de Santa Catarina (CEUA-UFSC, protocol number PP00956), and were carried out in accordance with the “Principles of Laboratory Animal Care” from NIH publication No. 85 - 23. Similarly, the experimental procedures were in agreement with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals, as previously specified (Zimmermann, 1983). The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects. Behavioral experiments were performed between 7:00 a.m. and 7:00 p.m.

2.2. Drug treatment protocol

Citral was stocked in amber glass, maintained at 4 °C and diluted in 5% Tween 80 solution made in saline (0.9% NaCl solution) to the desired concentration (50, 100 and 300 mg/kg) just before use. Citral or vehicle were orally administered by gavage (p.o.), 1 h before inflammatory stimulus and behavioral tests. Dexamethasone (0.5 mg/kg) was used as anti-inflammatory control and injected intraperitoneally (i.p.) one hour before intraplantar (i.pl.)

injections. The choice of dose drug based on previous data described in the literature (de Vasconcelos, et al., 2011; Nishijima, et al., 2014; Ponce-Monter, et al., 2010).

2.3. Anti-inflammatory activity

2.3.1. Carrageenan-induced hind paw edema model

To induce inflammation, mice received subcutaneous injections in the plantar surface of λ-carrageenan (2.5%) in 50 µl of saline (0.9% NaCl solution) in the left (ipsilateral) hind paw and 50 µL of saline in the right (contralateral) hind paw, as previously described (de Vasconcelos, et al., 2011). The paw edema was measured plethysmographically (model 7150, Ugo Basile, Varese, Italy) both before and at 1, 2, 3, 4, 6, 24 and 48 h after carrageenan injection. The data obtained were expressed in ml. The percentage inhibition was calculated based on the data of the area under the time – curves (AUC ∑ different time-points after i.pl injection) using the following formula *Inhibition = (1–T/V) x 100*, where T means test and V means vehicle.

2.3.2. LPS-induced paw edema

LPS, a TLR4 ligand, from *Escherichia coli* 026:B6 was dissolved in saline (0.9% NaCl solution). Acute inflammation of mouse hind paws was induced according to published methods (Kanaan, et al., 1996). For the edema studies, LPS (50 µg in 50 µl of saline – concentration of 1 µg/µl) was injected subcutaneously into the plantar region of the left hind paw, and saline (50 µl) was injected into the right hind paw. The edema of the LPS-treated and control

paws were measured both before and after LPS injection at the time points, as described above.

2.3.3. Zymosan-induced hind paw inflammation

To induce a stimulus for TLR2 and dectin-1, mice received i.pl. injections of zymosan A from *Saccharomyces cerevisiae* (1%) in 20 µl of saline (0.9% NaCl solution) in the left (ipsilateral) hind paw and 20 µl of saline in the right (contralateral) hind paw (de Vasconcelos, et al., 2011). The paw edema was measured both before and after zymosan injection at the time points, by means of plethysmometer, as described above.

2.4. Thermal allodynia test

Pain nociception was determined using the paw immersion test, as described previously (Silva, et al., 2013). Briefly, mice were gently handled and their right paw was dipped into a water bath at 38°C, which is considered a non-noxious heat stimulus. This low-intensity stimulus yields baseline latencies (15 s) that are long enough to observe hyperalgesia or analgesia. Thus, the latency paw withdrawal response within 15 s was considered to be nociceptive behavior. To determine the basal thermal allodynia thresholds, all the experimental groups were submitted to a pre-injection evaluation (basal assessment) and they were re-evaluated at several time-points. After 15 s, if the animals did not withdrawal their paw, the stimulus was suspended. The animals were acclimatized for 1 h before behavioural testing. All withdrawal latencies were measured manually, and the observer was fully blinded to the experimental protocol for all tests.

2.5. Drugs and reagents

Citral (95%), λ carrageenan, LPS (*Escherichia coli* 026:B6), zymosan A (from *Saccharomyces cerevisiae*) and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents used were all of analytical grade and obtained from different commercial sources.

2.6. Statistical analysis

Results are presented as means +/- SEM of measurements made on 4 – 6 mice/group/experiment. The results are representative of one or two independent experiments. A statistical comparison of the data was performed by one or two-way ANOVA followed by Bonferroni or Newman–Keuls testing, depending on the experimental protocol. P-values less than 0.05 were considered significant. The statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effect of citral on paw edema induced by carrageenan

In order to determine the anti-inflammatory activity of citral in acute-phase inflammation *in vivo*, carrageenan-induced paw edema model was conducted by intraplantar (i.pl.) injection of solution 2.5%. As expected, swelling of paw occurred rapidly after carrageenan-injection, with increase in volume at 1 h, which reached a maximum at 24 h (Fig. 2B and C). The oral treatment with citral (50, 100 or 300 mg/kg), 1 h beforehand, significantly inhibited the paw edema by i.pl. carrageenan injections (2.5%, 50 µl/paw), an effect that lasted for up to 24 h (Fig. 2B, C), with a percentage of inhibition of 57 ± 2.0%, 70 ± 4.0%

and 66 ± 3.5%, respectively, when compared with the carrageenan group (calculated on the basis of the area under the curve) (Fig. 1D). It is noteworthy that this effect was quite similar to that caused by dexamethasone (0.5 mg/kg, s.c.) – inhibition of 82 ± 5.5% – the clinical anti-inflammatory reference drug ($p<0.001$) (Fig. 2B D).

3.2. Citral inhibits thermal allodynia induced by carrageenan in mice

In order to investigate the possible anti-hypersalgesic effect of citral, we evaluated the thermal allodynia induced by paw injection of carrageenan model. The thermal allodynia produced following i.pl. carrageenan injections (2.5%, 50 µl/paw) was characterized by pronounced and long-lasting reduction on withdrawal latency from 15 ± 0.3 s at baseline for the naïve group to 8.0 ± 0.2 s for the carrageenan-group, especially, 15 min after i.pl. injection. This hypersalgesic behaviour was observed starting 15 min, reaching a maximum on 30 min after injection and remained elevated for up to 150 min after carrageenan-injection (Fig. 2A). Significantly, citral (50, 100 or 300 mg/kg, p.o.) was effective to reduce the thermal allodynia with an inhibition of 62 ± 8.0%, 80 ± 6.0% and 62 ± 3%, respectively, when compared with the carrageenan group, based on AUC graph (Fig. 2B). Similar to the results obtained with citral, the treatment with dexamethasone (0.5 mg/kg, s.c.) was able to reduce the thermal allodynia induced by carrageenan inhibitions of 72 ± 7% ($p<0.001$). Based on these results, a dose of 50 mg/kg of citral was used in subsequent experiments to investigate some of the mechanisms underlying its anti-inflammatory effect.

3.3. Citral exerts anti-inflammatory effects by modulating TLR4 pathway

LPS is a ubiquitous molecule found on the surface of Gram-negative bacteria and is recognized by innate immune cells in humans. Slightly elevated levels of LPS persist in humans with chronic diseases and lifestyles that involve chronic smoking and drinking (Manco et al., 2010; Mehta et al., 2010; Sun et al., 2010; Laugerette et al., 2011;). The first events in the immune response to LPS occur outside of the cell. LPS must first come into contact with the LPS-binding protein (LBP). The LPS–LBP complex can then be recognized by TLR4, acting in conjunction with MD-2 and CD14 (Park and Lee, 2013). Once this recognition has occurred, the TLR4 signaling cascade can commence, and upon ligation of TLR4 by LPS, signaling can proceed through a MyD88-dependent or MyD88-independent pathway. Herein, we assessed whether the treatment with citral could prevent LPS-induced paw-edema formation. The results in Figure 3 indicate that LPS (1 µg/µl, i.pl.) induced paw-edema, an effect that lasted for up 3 h (Fig. 3A). Of note, the acute oral administration of citral (50 mg/kg, p.o.), 1 h before inflammatory stimuli, consistently reversed paw-edema formation induced by LPS-injection (Fig. 3A), with an inhibition of 70 ± 6.0% ($p<0.001$), when compared with the LPS untreated-group, based on AUC graph (Fig. 3B). These data clearly show that even in presence of acute or intense inflammatory response, the oral administration of citral produced marked anti-inflammatory and analgesic effects, without any observable evidence of side effects.

3.4. Anti-inflammatory effect of citral is dependent on TLR2/dectin-1 signaling pathway

Zymosan, a yeast cell wall derivative, is recognized by dectin-1, a C-type lectin receptor for β-glucans (Brown et al., 2002, Reid et al., 2004) expressed in murine (Reid et al., 2004) and human (Yokota et al., 2001) DCs in conjunction with TLR2 (Gantner et al., 2003). In this set of experiments, we investigated whether the treatment with citral could inhibit the persistent inflammatory edema via TLR2/dectin-1-dependent mechanisms in mice. In the control group, i.pl. injection of zymosan (1%, 20 µl/paw) increased paw-edema formation, beginning 2 h after injection, reaching a maximum between 3-4 h, and remained elevated for up to 48 h after inflammatory stimulus (Fig. 4A). Notably, the oral pre-treatment of mice with citral (50 mg/kg), 1 h before, inhibited zymosan-induced paw edema (1%, 20 µl/paw), with inhibition of 45 ± 5.0% ($p<0.001$) (Fig. 4A and B). Dexamethasone (0.5 mg/kg, s.c.), the reference drug used in the clinics, exhibited essentially the same degree of anti-inflammatory action observed for citral (50 mg/kg, p.o.) (Fig. 4A and B). Taken together, these series of results suggest that anti-inflammatory and analgesic effect of citral is critically dependent on TLR4 and TLR2/dectin-1 modulation.

4. Discussion

Citral is well known as a major component (more than 70%) of lemongrass oil (Rabbani et al., 2006), and its anti-fungal, anti-bacterial, anti-oxidant and free radical scavenging activities have been described previously (Cheel et al., 2005; Lertsatitthanakorn et al., 2006). Herein, we investigated the anti-inflammatory and analgesic effects of citral in experimental models of acute

inflammation and nociception in mice, through modulation of eicosanoid- and TLR-depend pathways. Our results demonstrate that oral citral treatment displayed anti-inflammatory and analgesic effects, and these effects seem to be related to its TLR4 and TLR2/dectin-1 modulation.

It is now well established that i.pl. injection of carrageenan produces a long-lasting edema and hyperalgesic response through central sensitization in response to the release of several pro-inflammatory mediators, such as prostaglandins (PGs), cytokines/chemokines and reactive oxygen species (ROS), which in turn increase the sensitivity of peripheral and central sensory pathways (Basbaum and Woolf, 1999; Minami et al., 2006). The present study showed that preventive oral treatment with citral produced a prominent inhibition of the edema and thermal allodynia induced by carrageenan, an action that lasted for up to 6 h and 150 min, respectively. This result confirms and extends the study conducted by Francisco et al., in which inflammatory response was inhibited by *Cymbopogon citrates* (Francisco et al., 2011). In addition, Lin et al. showed that citral inhibited croton oil-induced mice ear edema, dosage of 0.1 and 0.3 mg/ear, with inhibition of 22 and 83%, respectively (Lin et al., 2008).

Lipopolysaccharide (LPS), a component of gram-negative bacteria walls, is a well-recognized TLR4 agonist used as inflammation-model (Cunha et al., 2008; Kawai and Akira, 2005; Sugama et al., 2009). Herein, our results showed that citral markedly inhibited LPS-induced paw edema, in accordance of Shen et al., which showed the protective effect of citral on LPS-induced acute lung injury (ALI) by activating PPAR-γ (Shen et al., 2015). Interestingly, this model is characterized by pulmonary edema, infiltration of neutrophils in the lung, and disruption of epithelial integrity (Karmpaliotis et al., 2002; Lucas et al., 2009).

PPAR-γ agonists have been reported to inhibit LPS-induced NF-κB activation, by inhibiting the phosphorylation of Iκ-B, which in turn inhibited p65 and p50 translocation to the nucleus – an initial process for gene expression of several cytokines, such as TNF-α, IL-1β and IL-6 involved in the process of acute lung injury (Blackwell, 1997; Chen et al., 2007; Larsson et al., 1999; Lee et al., 2007; Parsons et al., 2005). Taken together, we suggest that a protective effect of citral on inflammation is associated, in part, with TLR4-pathways inhibition which possibly infer in the inhibition of proinflammatory mediators and activation of PPAR-γ pathway. However, future studies will need to clarify this hypothesis, as well as to investigate whether citral could inhibit TLR4 through a MyD88-dependent or MyD88-independent pathway.

Zymosan stimulates phagocytosis, the release of inflammatory cytokines/chemokines, upregulation of reactive oxygen and nitrogen species, as well as stimulates adaptive immune responses through TLRs pathway (Underhill, 1999; Gold, 2000). Following macrophage or DCs recognition of zymosan, both *i)* dectin-1, a lectin family receptor for β-glucans, and *ii)* TLR2/CD14 are recruited to phagosomes, which binds distinct components of the yeast cell wall and induces immune response. Previously studies have demonstrated that TLR stimulation leads to activation of NF-κB and production of proinflammatory cytokines such as TNF-α and IL-12 (Medzhitov, 1997; Reid et al., 2004). However, there are no reports on the effect of citral on models of inflammation induced by zymosan. Here, our findings revealed that the inflammation caused by zymosan was also greatly inhibited following oral pre-treatment with citral. As published previously, citral inhibited expression of inducible nitric oxide synthase (iNOS) and NF-κB (Lin et al., 2008), as well as

release of IL-1β and IL-6, leukocytes migration (Bachiega et al., 2011). Alternatively, it is possible to suggest that citral inhibits inflammatory response through TLR2/dectin-1-pathways inhibition associated with its ability to interfere with the release and/or expression of various relevant inflammatory and pain mediators and neutrophils migration. Nonetheless additional studies are necessary to test this hypothesis.

In addition, we demonstrate that citral was effective to reduce thermal allodynia. A previous study demonstrated that citral has an anti-nociceptive effect when administered via i.p. route (Quintans-Junior et al., 2011). Moreover, Caterine and colleagues provides, the first evidence, of the anti-nociceptive effect of citral administered by oral route in acute and chronic pain model. Glutamate is a major excitatory neurotransmitter that transmits nociceptive signals by promoting the direct activation of receptors in nociceptive fibres. Once activated, these neurons release several inflammatory mediators and neuropeptides, which are involved in nociceptive transmission in the central and peripheral nervous systems (Fundytus, 2001; Millan, 1999). Thus, citral administered orally evoked an anti-nociceptive effect against the nociception induced by peripheral injection of glutamate, suggesting that this effect could occur through a direct action of citral on glutamatergic neurons in the spinal cord, which possibly correlates with our findings that the citral has analgesic properties, reducing the thermal allodynia when compared with the carrageenan group. Additional experiments are necessary to confirm whether or not the citral can selectively inhibit glutamatergic primary neurons in the spinal cord and dorsal root ganglion.

In summary, data presented herein demonstrate that citral has anti-inflammatory and analgesic activity in the rodent paw edema model induced by carrageenan, zymosan and LPS, suggesting a possible interaction with eicosanoid, TLR4 and TLR2/dectin-1 pathways, both involved in the innate immune response.

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Conflict of interest

All authors report no conflict of interest.

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Figures

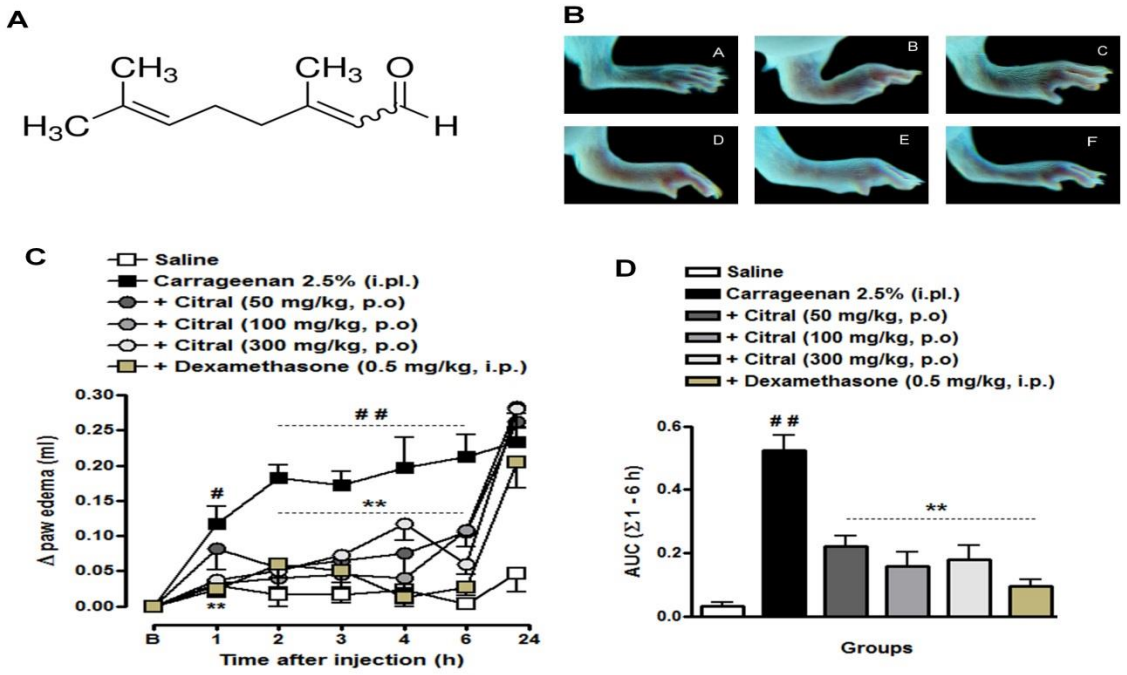

Figure 1. Effect of citral on carrageenan-induced paw edema in mice. (A) Chemical structure of the open-chain monoterpenoid citral (which is a mixture of 2 isomers (*cis*-isomer neral and *trans*-isomer geranial). Representative photograph (B), graphical representation of the paw edema (C) and area under curve (D) from 1, vehicle-treated control; 2, carrageenan-untreated; carrageenan plus: 3, citral (50 mg/kg, p.o.); 4, citral (100 mg/kg, p.o.); 5, citral (300 mg/kg, p.o.); 6, dexamethasone (0.5 mg/kg, s.c.). Citral and dexamethasone were administered 1 h prior to carrageenan (2.5%) injection, and the mice were evaluated for paw edema at 1, 2, 3, 4, 6, 24 and 48 h post- carrageenan injection. Data are presented as mean ± SEM of 4–6 mice/group. $^{\#}p<0.05$ and $^{\#\#}p<0.001$ versus vehicle control group, *p<0.05 and **p<0.001 versus carrageenan-untreated group (two-way ANOVA followed by Bonferroni's test to A, and one-way ANOVA followed by Newmann–Keuls test to D).

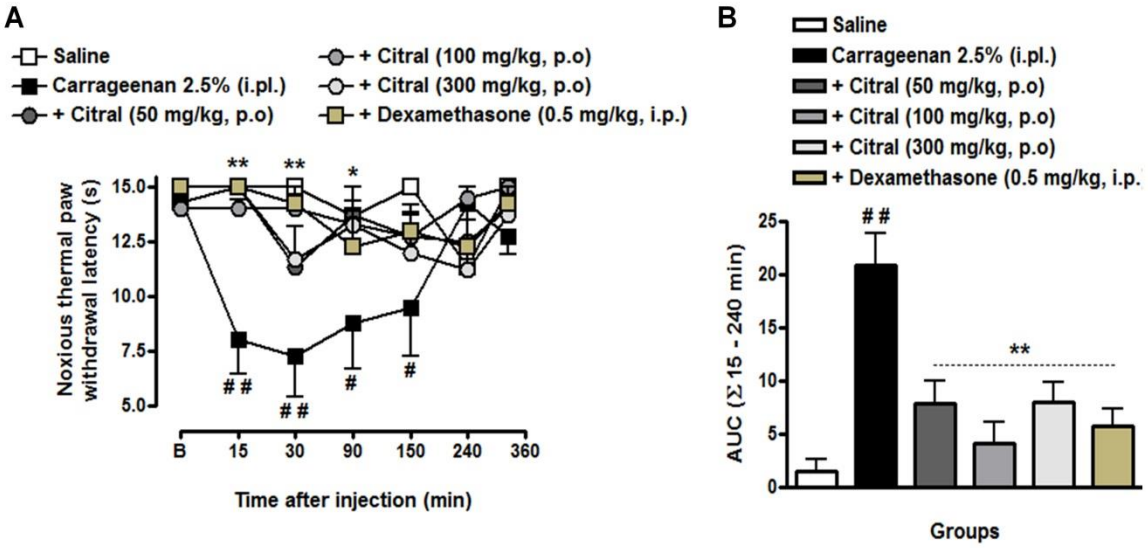

Figure 2. Effects of citral on thermal allodynia induced by carrageenan. Time- course for the effects of citral (50, 100 or 300 mg/kg, p.o.) and dexamethasone (0.5 mg/kg, s.c.) on thermal allodynia (A) and area under curve induced by carrageenan (2.5%) injection. Data are presented as mean ± SEM of 4–6 mice/group. #p<0.05 and ##p<0.001 versus vehicle control group, *p<0.05 and **p<0.001 versus carrageenan-untreated group (two-way ANOVA followed by Bonferroni‟s test to A, and one-way ANOVA followed by Newmann–Keuls test to B).

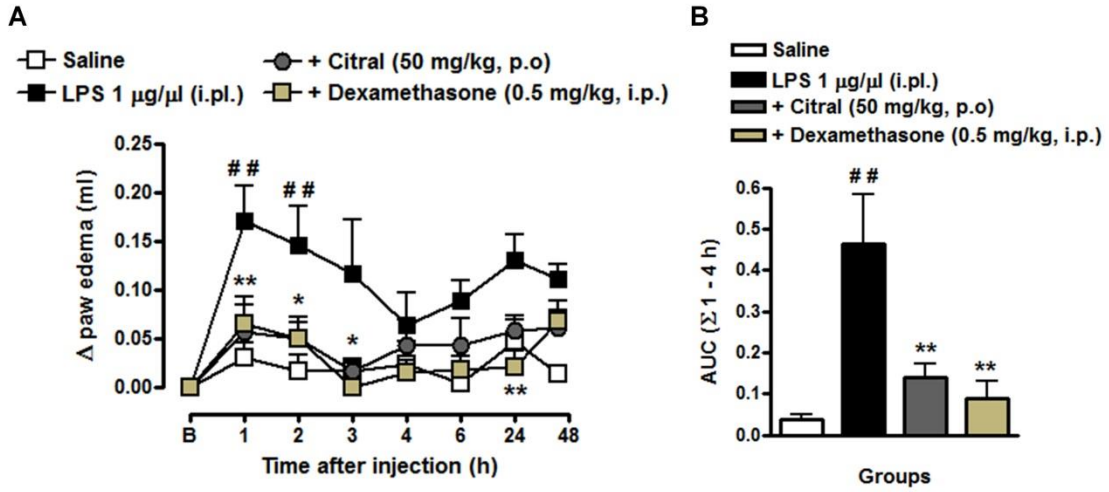

Figure 3. Effect of citral on LPS-induced paws edema in mice. Time-course for the effects of citral (50 mg/kg, p.o.) and dexamethasone (0.5 mg/kg, s.c.) on paw edema (A) and area under curve induced by LPS (50 µg in 50 µl of saline – concentration of 1 µg/µl) injection. Data are presented as mean ± SEM of 4–6 mice/group. ##p<0.001 versus vehicle control group, *p<0.05 and **p<0.001 versus carrageenan-untreated group (two-way ANOVA followed by Bonferroni"s test to A, and one-way ANOVA followed by Newmann–Keuls test to B).

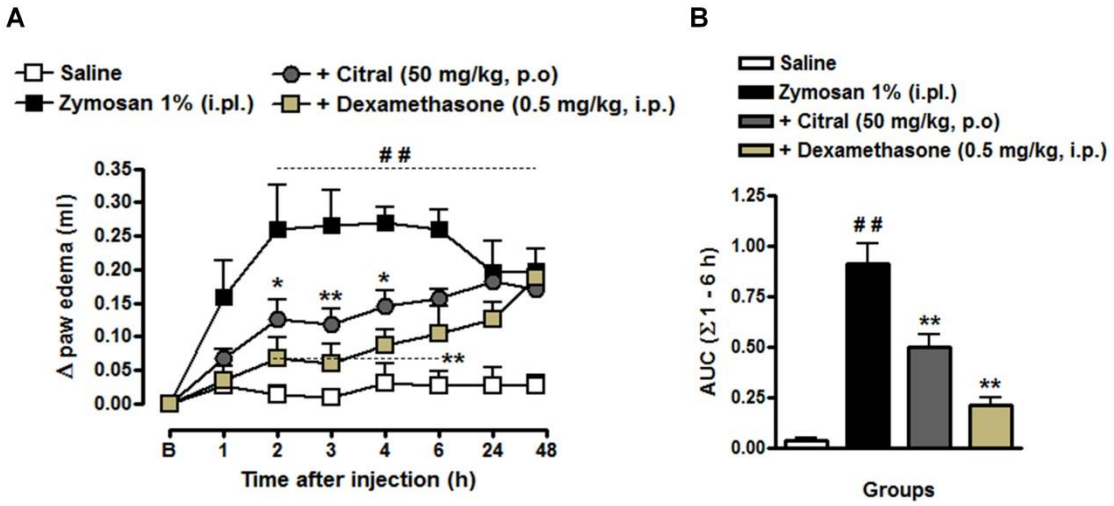

Figure 4. Effect of citral on zymosan-induced paw edema in mice. Time-course for the effects of citral (50 mg/kg, p.o.) and dexamethasone (0.5 mg/kg, s.c.) on paw edema (A) and area under curve induced by zymosan (1%) injection. Data are presented as mean ± SEM of 4–6 mice/group. ##p<0.001 versus vehicle control group, *p<0.05 and **p<0.001 versus carrageenan-untreated group (two-way ANOVA followed by Bonferroni‟s test to A, and one-way ANOVA followed by Newmann–Keuls test to B).